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Title of the Invention: method for Measuring

Complex of activated human protein C with human protein C inhibitor and measuring reagent therefor.

Specification

1. Title of the Invention:

Method for Measuring complex of Activated Human Protein C with Human Protein C Inhibitor and Measuring Reagent Therefor

2. Scope of Claims for a Patent:

1. A method for measuring a complex of Activated Human Protein C with Human Protein C Inhibitor by an enzyme immunoassay utilizing a double antibody sandwich technique, characterized by using an anti-human protein C inhibitor monoclonal antibody as a solid phase-coating antibody and an anti-human protein C monoclonal antibody as an enzyme-labeled antibody to participate in said measurement.

2. A reagent for measuring a complex of Activated Human Protein C with Human Protein C Inhibitor by an enzyme immunoassay utilizing a the double antibody sandwich technique, characterized by comprising an anti-human protein C inhibitor monoclonal antibody as a solid phase-coating antibody and an anti-human protein C monoclonal antibody as an enzyme-labeled antibody to participate in said measurement.

3. A reagent according to Claim 2, which works as a diagnostic reagent for the disseminated intravascular coagulation syndrome.

3. Detailed Description of the Invention:

(Technical Field to Which the Invention Pertains)

The present invention relates to a method for measuring a complex of Activated Human Protein C with Human Protein C Inhibitor (hereinafter referred to as APC-PCI Complex or

CIC) and a measuring reagent therefor. More particularly, it relates to a method for measuring an APC-PCI Complex (CIC) by means of the enzyme immunoassay with the use of the double antibody sandwich technique and a measuring reagent therefor.

(Prior Art and Problems to be Solved by the Invention)

Human proteins C (hereinafter referred to as PC) are one of Vitamin K dependent plasma proteins, comprising a double-strand having a 2% sugar content, having a molecular weight of about 62000 and playing an important role as a physiological control factor of blood coagulation. Ordinarily, PC occurs as a precursor enzyme of inactive type in the circulating blood. Once the coagulation system begins to work and produce thrombin for whatsoever reasons, it combines with thrombomodulin on the surface layer of the vascular endothelium cells, forming a thrombin-thrombomodulin complex. This thrombin-thrombomodulin complex changes PC into activated human proteins C (hereinafter referred to as APC). APC controls the coagulation reaction by breaking down Activated Factors VIII and V as a co-enzyme of the coagulation reaction.

A protein C inhibitor (hereinafter referred to as PCI) occurs as an inhibitor against APC in the blood. The PCI is a single-stranded glycoprotein having a molecular weight of 57000, capable of inhibiting APC by forming a complex with APC by means of a bond of equimolecular acyls.

It has been reported that human blood PC can be measured either by a method in terms of biological activity or by an immunological method. To measure the PCI, a biological activity method and an immunological method have

been established. However, any useful and practical methods and reagents have not as yet been provided, in order to measure the APC-PCI Complex. As an example of the methods for purifying and measuring PC and the PCI, References 1) to 3) are listed below:

1) Suzuki K. Kishioka J. Hashimoto S.,
Protein C Inhibitor, Purification from Human Plasma and Characterization,
J. Biol. Chem., 1983:258:163

2) Suzuki K. Stenflo J. Dahlback B. Teodorsson B.,
Inactivation of Human Coagulation Factor V by Activated Protein C,
J. Biol. Chem., 1983:258:1918

3) Suzuki Kohji,
Protein C,
Clinical Examination, 1984:28:25

The disseminated intravascular coagulation syndrome (hereinafter referred to as DIC) is a disease wherein many thrombi are generated mainly in the small blood vessels systemically. The DIC is ordinarily accompanied by a hemostatic disorder, disorders in various organs due to thrombus, a shock and a failure of peripheral blood circulation. The other underlying diseases cause the DIC, and malignant tumors account for the highest ratio of these concomitant diseases at 45.2%, followed by infections, leukemia and hepatic disorders in the mentioned order. An early discovery and early treatment is the best way of dealing with the DIC as the complication to the underlying diseases.

At present, measuring the number of platelet, the prothrombin time, plasma fibrinogen and the serum FDP (a

fibrin-fibrinogen decomposition product) screens the DIC. Measuring plasma antithrombin Ⅲ, plasma α_2 and the plasmin inhibitor also makes an auxiliary diagnosis. Furthermore, it has been found that PC and the PCI are at a low side in the DIC patients, as compared with those of the healthy people.

However, these screening methods can hardly make the diagnosis at an early stage of the DIC, and there has been a demand for a clinical examination method capable of the early diagnosis.

(Means for Solving the Problems)

In consideration of these circumstances, the present inventor has conducted an investigation, in an attempt to obtain a practical method capable of measuring the APC-PCI Complex (CIC) expediently and particularly handling a number of test samples at the site of clinical examination at a time. As a result, it has been found that the problems can find a solution by implementing a double antibody sandwich enzyme immunoassay using an anti-human protein C monoclonal antibody (hereinafter referred to as anti-PC antibody) and an anti-human protein C inhibitor monoclonal antibody (hereinafter referred to as anti-PCI antibody for short). It has also been found that the measuring reagent of the present invention makes it possible to diagnose the DIC at its early stage. The present invention has been completed on the basis of these findings.

Namely, the present invention provides a method for measuring an APC-PCI Complex (CIC) by the enzyme immunoassay utilizing the double antibody sandwich technique, characterized by

using an anti-PCI antibody as a solid phase-coating antibody and an anti-PC antibody as an enzyme-labeled antibody to participate in said measurement. The present invention also provides a reagent for measuring an APC-PCI Complex (CIC) by the enzyme immunoassay with the aid of the double antibody sandwich technique, characterized by comprising an anti-PCI antibody as a solid phase-coating antibody and an anti-PC antibody as an enzyme-labeled antibody to participate in said measurement.

The present invention will be described in detail below.

An anti-APC antibody reactive with APC and an anti-PCI antibody reactive with PCI are both necessary to measure the APC-PCI Complex (CIC) by the double antibody sandwich enzyme immunoassay. Both APC and PC have common antigenicity, permitting the use of the anti-PC antibody.

The anti-PC antibody of the present invention is manufactured, for example, in the following way. But the anti-PC antibody obtainable from market may as well be used.

At first, PC is prepared. For this, the human plasma is added with barium salt. After the precipitate is formed, PC is eluted from it with ethylenediamine tetraacetate (EDTA), and eluted PC is purified with DEAE-Sephacel and Heparin-Sepharose. The purification method is described in detail in Reference 2 as listed above.

Fifty μg of so prepared PC is intraperitoneally administered to BALB/c mice, together with the same dose of complete Freund's adjuvant. After 2 weeks, further 15 μg of PC is intravenously administered to the tail of the mice and 3 days later the spleen cells are harvested from them. The cells are fused with the myeloma

cell strain P3UI according to the method of Köhler and Milstein (See Reference 4) as listed below). Thereafter, the hybridoma is cloned three times by the limiting dilution analysis, establishing an anti-PC antibody-formed cell line.

The monoclonal anti-PCI antibody of the present invention is manufactured in the following way.

At first, PCI is prepared. For this, the human plasma is added with barium salt, and Vitamin K dependent proteins such as PC are removed from the mixture. To the supernatant thereof, PEG-6000 is added to collect the precipitates. Then, the precipitates are subjected to the elution, and the eluted PCI is purified with DEAE-Sepharose, ammonium sulfate fractionation, Dextran Sulfate-Agarose Chromatography, Ultrogel AcA44 and DEAE-Sephacel Chromatography. The purification method is described in detail in Reference 1) as listed above.

Fifty μ g of the so prepared PCI is intraperitoneally administered to BALB/c mice, together with the same dose of complete Freund's adjuvant. After 2 weeks, further 15 μ g of the PCI is intravenously administered to the tail of the mice and 3 days later the spleen cells are harvested from them. The cells are fused with the myeloma cell strain P3UI according to the method of Köhler and Milstein (See Reference 4) as listed below). Thereafter, the hybridoma is cloned three times by the limiting dilution analysis, establishing an anti-PCI antibody-formed cell line.

4) Köhler G. Milstein G.,
Deviation of specific antibody-producing culture and
and tumor lines by cell fusion,
Eur. J. Immunol. 1976:6:511

In the present invention, the enzyme immunoassay measures the APC-PCI Complex (CIC) by using the double antibody sandwich technique in the following way.

As the whole, the measurement system comprises a solid phase, a solid phase-coating anti-PCI antibody (1st antibody), a standard antigen, and an anti-PC antibody to be labeled (2nd antibody), an enzyme and a substrate. As the solid phase, wells of microtiter plate for enzyme immunoassay or beads of plastics such as polystyrene can be used. Prior to the measurement, the anti-PCI monoclonal antibodies are dissolved in a carbonic acid buffer solution, and the mixed solution is allowed to stand overnight at 4℃ to coat the surface of the solid phase. However, parts of the surface may remain uncoated with some of the monoclonal antibodies. These uncoated parts are coated with bovine serum albumin in the following way. Bovine serum albumin is dissolved by the addition of a phosphoric acid buffer solution, the mixed solution is added to the wells and the wells are allowed to stand for 2 hours at room temperature to complete the bovine serum albumin coating.

The enzyme-labeled anti-PC antibodies (the anti-PC antibody obtainable from BioScots Corporation-Distributor Cosmo Bio Co., Ltd. under the tradename of "Anti-Human Protein C") are manufactured in the following way. The enzyme to be used herein is alkaline phosphatase, glucose oxidase, peroxidase, β-galactosidase and the like. Prior to the measurement, the enzyme is bonded to the anti-PC antibody with glutaraldehyde or a maleimide compound, forming a conjugate. The conjugate can previously be held in store as part of the reagents until they are

used in implementing the measuring method of the present invention. As the bonding method, for example, the maleimide method described in Reference 5) as listed below can be applied to label the antibody.

5) Yoshitake S. Imagawa M. Ishikawa E., et al.,
Mild and Efficient Conjugation of Rabbit Fab' and
Horseradish Peroxidase Using a Maleimide Compound
and Its Use for Enzyme Immunoassay

The present invention uses a suitable substrate matching the selection of enzyme. For example, if alkaline phosphatase is selected as the enzyme, the substrate is p-nitrophenyl phosphate. If peroxidase is selected as the enzyme, the substrate is hydrogen peroxide with the use of o-phenylendiamine or ABTS (2,2'-azino-bis(3'-ethylbenzothiazoline sulfonic acid) as a color producing reagent

The APC-PCI Complex (CIC) is measured in accordance with the procedure established for the enzyme immunoassay using the double antibody sandwich technique. Prior to the measurement, the test samples such as serum and plasma are pre-treated by the addition of barium salt. The mixture is stirred for 1 hour following the addition and is subjected to the centrifugation to obtain the precipitate. As a result of this operation, PC, APC and the APC-PCI Complex (CIC) containing γ -carboxyglutamic acid are combined with barium salt and the resultant mixture is precipitated, while a greater part of unreacted PCI from the test sample remain in the supernatant, separated from the APC-PCI Complex (CIC). If this operation does not take place, most unreacted PCI reacts with the anti-PCI antibody in the solid phase, with the result that the

measurement of the APC-PCI Complex (CIC) is unfeasible. The precipitate is dissolved by the addition of an EDTA-containing Tris buffer solution to complete a test specimen. The test specimen is measured by adding it to the solid phase coated with the anti-PCI antibody and incubating them as illustrated in the Examples, which will be described below. After the solid phase is washed, the enzyme-labeled anti-PC antibody is added to the solid phase, followed by the incubation and washing again. Finally the substrate is added to the wells and the so loaded wells are incubated. After the reaction is brought to an end, the amount of decomposed substrate is measured by a spectrophotometer.

The measuring reagent of the present invention is a reagent designed to be used directly in implementing the measuring method of the present invention and to achieve the same object as in the measuring method. Accordingly, the measuring reagent of the present invention is specifically embodied in the following way.

The measuring reagent of the present invention comprises a solid phase-coating anti-PCI antibody and an enzyme-labeled anti-PC antibody as the essential components. It is within one's discretion to design a set of these essential components, together with suitable materials convenient to implement the measurement, such as standard antigen, pre-treatment agent, antigen diluent, reactive solution, substrate, substrate-dissolving liquid and reaction-stopping liquid. However, the present invention will by no means be limited to this arrangement.

As illustrated in the Experimental Examples, which will be described later, the measuring reagent of the present invention makes it possible to diagnose the DIC at its early stage and thus

can be used as a DIC diagnostic reagent.

The effects of the present invention can be summarized as follows:

At first, the present invention can measure the APC-PCI Complex (CIC) by the enzyme immunoassay with the use of the double antibody sandwich technique, although the measurement thereof has conventionally been regarded as impossible. The method can be operated simply and concisely, having high serviceability at the sites of clinical examination. Furthermore, the method makes it possible to diagnose the DIC or deep phlebothrombosis at their early stage.

〔Examples〕

The present invention will be described in greater detail with reference to the following Examples.

Example 1:

1) To 4 l of the fresh plasma, benzamidine hydrochloride (10 mM), diisopropyl fluorophospate (DFP)(1 mM), phenylmethylsulfonyl fluoride (PMSF)(1 mM) and soybean trypsin inhibitor (50 mg/l) were added, and then 320 ml of 1M $BaCl_2$ was added dropwise to the mixture. The following operations were all conducted at 4 ℃. After the mixture was stirred for 1 hour, it was centrifuged at 5000 rotations for 30 minutes, and the supernatant was withdrawn and 60 g/l of solid PEG-6000 was added to the supernatant. After the mixture was stirred for 1 hour, it was centrifuged at 5000 rotations for 15 minutes, and the precipitate was discarded. Furthermore, 60 g/l of solid PEG-6000 was added to the supernatant, and after the mixture was stirred for 1 hour, it was centrifuged at 5000 rotations for 30 minutes and the precipitate was withdrawn. The

PCI purif

precipitate was dissolved by the addition of 500 ml of 0.05M Tris-hydrochloric acid buffer solution at pH 7.5 (0.1M NH_4Cl, benzamidine hydrochloride (10 mM), DFP (1 mM) and PMSF (1 mM)). The solution was placed in a DEAE-Sepharose CL-6B column equilibrated with the same buffer solution as used in the dissolution, and the passing-through fraction was withdrawn. The withdrawn liquid was 50% saturated by the addition of ammbnium sulfate powder. After the mixture was stirred for 1 hour, it was centrifuged at 8000 rotations for 15 minutes, and the supernatant was withdrawn. The supernatant was 70% saturated by the addition of ammonium sulfate powder. After the mixture was stirred for 1 hour, it was centrifuged at 8000 rotations for 30 minutes, and the precipitate was withdrawn. The precipitate was dissolved by the addition of 0.05M Tris-hydrochloric acid buffer solution at pH 7.0 (0.1M NaCl, benzamidine hydrochloride (1 mM), DFP (0.1 mM) and PMSF (0.1 mM)), and the solution was dialyzed with the same buffer solution. Then, the dialyzate was placed in a Dextran Sulfate-Agarose column, and thereafter the PCI fraction was 80% saturated by the addition of ammonium sulfate powder. The mixture was centrifuged at 10000 rotations for 15 minutes to obtain the precipitate. The precipitate was dissolved by the addition of 0.05M Tris-hydrochloric acid buffer solution at pH7.5 (0.15 M NaCl) in a least dissolvable amount, and the solution was placed in an AcA-44 Ultrogel column, and the PCI fractions were collected and dialyzed with the 0.05M Tris-hydrochloric acid buffer solution at pH9.0. After the dialyzation, the dialyzate was placed on a DEAE-Sephacel column, and in this way the purified PCI was obtained. The final yield thereof was found to be 9%.

PCI purify

2) Fifty μg of the purified PCI was intraperitoneally administered to BALB/c mice (female, 8 weeks old), together with the same dose of complete Freund's adjuvant. After 2 weeks, further 15 μg of the PCI was intravenously administered to the tail of the mice, and 3 days later the spleen cells were extracted from them. The spleen cells were fused with the myeloma cell strain P3Ul. The cell fusion was conducted with polyethylene glycol 4000 according to the Köhler and Milstein method (Reference 4)). Then, the hybridoma reactive with the PCI was cloned three times by the limiting dilution analysis with the use of a 96 well microplate, establishing an anti-PCI antibody-formed cell line. The preservation medium used for the cell line was a RPMI1640 medium containing 10% of fetal bovine serum. The monoclonal anti-PCI antibody was obtained from the cell line in the usual way.

3) The so obtained anti-PCI antibody was diluted with 0.1M carbonic acid buffer solution at pH 9.3 to make 3 μg/ml, and 100 μl each of the diluted anti-PCI antibody was injected per well of the 96 well microplate and the so loaded wells were allowed to stand overnight at 4°C. The wells were washed three times with 0.05M Tris-hydrochloric acid buffer solution at pH7.5 (0.2M NaCl, 0.5% bovine serum albumin, 0.05% Tween-20, 0.05 mM EDTA, 0.02% thimerosal). Thereafter, 150 μl of 5% bovine serum albumin (0.5% gelatin, phosphoric acid buffer solution at pH7.5) was injected per well, and the so injected wells were allowed to stand for 2 hours and then were washed three times with said buffer solution. In this way, the antibody-coated solid phase was completed.

Example 2:

1) To 4.4 l of the fresh plasma, benzamidine hydrochloride (10 mM),

DFP(1 mM), PMSF(1 mM) and soybean trypsin inhibitor (50 mg/l) were added, and then 350 ml of 1M $BaCl_2$ was added dropwise to the mixture. The following operations were all conducted at 4 ℃. After the mixture was stirred for 1 hour, it was centrifuged at 5000 rotations for 30 minutes, and the precipitate was withdrawn. The precipitate was washed two times by the addition of 700 ml of 0.15M NaCl (5 mM benzamidine hydrochloride) at pH 7.4. Proteins adsorbed to barium salt were eluted by the addition of 660 ml of 0.2M EDTA at pH7.4 (5 mM benzamidine hydrochloride, 0.1 mM DFP). After the suspension was stirred for 1 hour, it was centrifuged at 5000 rotations for 30 minutes, to remove the precipitate. The supernatant was dialyzed in 0.1M phosphoric acid buffer solution at pH 6.0 (1 mM benzamidine hydrochloride). Then, the dialyzate was placed in a DEAE-Sephacel column equilibrated with the same buffer solution as used in the dialyzation, to elute and collect the PC fraction in a linear concentration gradient from 0.1 M to 0.7 M NaCl. The collected liquid was dialyzed with 0.05 M Tris-hydrochloric acid buffer solution at pH8.0 (1 mM benzamidine hydrochloride). After the dialyzation, DFP was added to make 1 mM and PMSF was added to make 0.1 mM in the dialyzate. Then, the mixture was placed in a DEAE-Sephacel column equilibrated with the 0.05 M Tris-hydrochloric acid buffer solution at pH8.0 (1 mM benzamidine hydrochloride), to elute and collect the PC fraction in a linear concentration gradient from 0 M to 0.5 M NaCl (0.05 M Tris-hydrochloric acid buffer solution at pH8, 0.1 mM benzamidine hydrochloride, 2 mM $CaCl_2$). The collected liquid was dialyzed with 50 mM imidazole buffer solution at pH6.0 (1 mM benzamidine hydrochloride). The precipitate formed by the

Prot purif

dialyzation was removed by the centrifugation at 20000 rotations for 10 minutes. $CaCl_2$ was added to the supernatant to make 2 mM. The mixture was placed in a Heparin-Sepharose column equilibrated with 50 mM imidazole buffer solution at pH 6.0 (1 mM benzamidine hydrochloride, 2 mM $CaCl_2$), to elute and collect purified PC in a linear concentration gradient from 0 M to 0.8 M NaCl (50 mM imidazole buffer solution at pH6.0, 1 mM benzamidine hydrochloride). The final yield of purified PC was found to be 25%.

2) Fifty μg of purified PC was intraperitoneally administered to BALB/c mice (female, 8 weeks old), together with the same dose of complete Freund's adjuvant. After 2 weeks, further 15 μg of PC was intravenously administered to the tail of the mice, and 3 days later the spleen cells were extracted from them. The spleen cells were fused with the myeloma cell strain P3UI. The cell fusion was carried out with polyethylene glycol 4000 according to the Köhler and Milstein method (Reference 4)). Then, the hybridoma reactive with PC was cloned three times by the limiting dilution analysis with the use of a 96 well microplate, establishing an anti-PC antibody-formed cell line. The preservation medium used for this cell line was a RPMI 1640 medium containing 10% of fetal bovine serum. The monoclonal anti-PC antibody was obtained from the cell line in the usual way.

3) Five mg of the anti-PC antibody was dialyzed with 0.1 M acetic acid buffer solution at pH 4.2, and then 0.2 mg of swine stomach pepsin was added to the dialyzate and the mixture was incubated at 37 ℃ for 24 hours. After the so incubated mixture was adjusted to have pH of 7.0, it was placed in an Ultrogel AcA44 column,

subjecting it to the gel filtration with 0.1 M phosphoric acid buffer solution at pH 7.0 and thus obtaining F $(ab')_2$. F $(ab')_2$ was dialyzed in 0.1 M phosphoric acid buffer solution at pH 6.0, and then 50 μ l of 0.1 M mercaptoethylamine (0.1 M phosphoric acid buffer solution at pH 6.0, 5 mM EDTA) was added to the dialyzate, and the mixture was incubated at 37℃ for 90 minutes. The Incubated mixture was dialyzed by passing it through a Sephadex G25 column equilibrated with 0.1 M phosphoric acid buffer solution at pH 6.0 (5 mM EDTA), in order to obtain Fab-SH. On the other hand, 2 mg of enzyme horseradish peroxidase (hereinafter called HRP for short) was dissolved in 0.1 M phosphoric acid buffer solution at pH 7.0. The resultant solution was added with 0.7 mg. of N-succinimidyl -4-(N-maleimidemethyl)-cyclohexane-1-carboxylate (dissolvable in N, N-dimethylformaldehyde), and the mixture was incubated at 30℃ for 60 minutes. The incubated mixture was dialyzed by passing it through a Sephadex G25 column equilibrated with 0.1 M phosphoric acid buffer solution at pH 6.0, and as a result, maleimide-combined HRP was obtained. Fab-SH and maleimide-combined HRP were mixed each other, and the resultant mixture was incubated at 4℃ overnight. The so incubated mixture was subjected to the gel filtration in an Ultrogel AcA44 column equilibrated with 0.1 M phosphoric acid buffer solution at pH 6.5. In this way, the HRP-labeled anti-PC antibody was obtained.

Ab-HRP

The antibody-coated solid phase obtained in Example 1 and the enzyme-labeled antibody obtained in Example 2 were assorted into a set of measuring reagent of the present invention.

Example 3:

The serum or plasma under test was pre-treated in the following way. To 150 μl of the serum or plasma test sample, 600 μl of 0.38% sodium citrate (0.1 M Tris-hydrochloric acid buffer solution at pH 7.5, 0.15 M NaCl) was added and then 60 ml of $BaCl_2$ was added dropwise to the mixture. After the mixture was stirred under the ice-cold condition for 1 hour, it was centrifuged at 15000 rotations for 5 minutes, to obtain the precipitate. The preqipitate was dissolved by the addition of 50 μl of 0.2 M EDTA (0.1 M Tris-hydrochloric acid buffer solution at pH 7.5, 0.15 M NaCl). To this solution, 250 μl of 0.05 M Tris-hydrochloric acid buffer solution at pH 7.5 (0.2 M NaCl, 0.5% bovine serum albumin, 0.05% Tween-20, 0.05 mM EDTA, 0.02% thimerosal) was added as an assay buffer solution. Of this mixed liquid, 100 μl would be used as a specimen under test.

Plasma incl.

Example 4:

The specimen under test as described in Example 3 is injected into the antibody-coated solid phase of Example 1 in an amount of 100 μl per well and the so injected solid phase is incubated at room temperature overnight. The wells are washed three times with 0.05 M Tris-hydrochloric acid buffer solution at pH 7.5 (0.2 M NaCl, 0.5% bovine serum albumin, 0.05% Tween-20, 0.05 mM EDTA, and 0.02% thimerosal). Thereafter, 100 μl of the enzyme-labeled antibody of Example 2 is added to the wells, and the so loaded wells are incubated at room temperature for 60 minutes. The incubated wells are washed three times with 0.05 M Tris-hydrochloric acid buffer solution at pH 7.5 (0.2 M NaCl, 0.5% bovine serum albumin, 0.05% Tween-20, 0.05 mM EDTA, 0.02% thimerosal), and then 100 μl of o-phenylenediamine having a

TRH assay

concentration of 2 mg/ml (citric acid buffer solution at pH 4.65, 0.03% hydrogen peroxide) was added thereto. The so processed specimen under test is allowed to stand at room temperate for 30 minutes, and thereafter its absorbance at the wavelength of 490 nm is measured by a spectrophotometer.

(Effects of the Invention)

The effects of the present Invention will be described below with reference to the following Experimental Examples.

Experimental Example 1:

Material and Method

Four-tenths ml of 1 M $BaCl_2$ was added per ml of the normal human plasma, and the mixture was stirred under the ice-cold condition for 60 minutes, removing the APC-PCI Complex (CIC) from the plasma by adsorption, and the resultant plasma was designated as the test sample a. Previously, APC had been prepared by activating PC obtained in said Example 2. Then, so obtained APC had been reacted with the PCI of Example 1, to provide a standard APC-PCI Complex (CIC). This standard APC-PCI Complex (CIC) was added to the test sample a to make 160 ng /ml, and in this way the standard antigen solution was completed. The standard antigen solution was subjected to the measurement by repeating the procedure of said Examples 3 and 4.

Results

The results are shown in Fig. 1. In Fig. 1, the axis of abscissa represents the concentration of APC-PCI Complex (CIC) in the specimen under test and the axis of ordinates represents the absorbance at the wavelength of 490 nm. It is evident from Fig. 1 that the present invention is highly specific to the APC-PCI

Complex (CIC) and excellent in calibrating characteristics.

Experimental Example 2:

Material and Method

The APC-PCI Complex (CIC) was measured in 50 plasma samples from the DIC patients, 19 plasma samples from the patients placed on a continuous Warfarin dose, 10 plasma samples from the hepatic disorder patients and 20 plasma samples from the healthy adult subjects. The measurement was conducted by repeating the procedure of said Examples 3 and 4.

Results

The results are shown in Fig. 2. In Fig. 2, the leftmost column represents the plasma of the healthy adult subjects, and the measured values of their APC-PCI Complex (CIC) were 0.57 ng/ml on average. In the plasma of the patients on the continuous Warfarin dose and the hepatic disorder patients, the measured values of their APC-PCI Complex (CIC) were respectively 0.37 ng/ml and 1.64 ng/ml on average. On the contrary, the measured values of the APC-PCI Complex (CIC) were found to be at a high side of 3.23 ng/ml on average in the plasma of DIC patients, showing that the method of the present invention is useful in the diagnosis of DIC.

Experimental Example 3:

Material and Method

An acute anterior myeloic leukemia patient was placed under observation for a long term of 40 days, wherein FDP, PCI and PC were measured and at the same time the APC-PCI Complex (CIC) was also measured by repeating the procedure of said Examples 3 and 4.

Results

The results are shown in Fig. 3. The measured values of the APC-PCI Complex (CIC) were found to be at a high side at an early stage of observation, as compared with the measured values of the coagulation test, with the resultant finding that the method of the present invention can make an early diagnosis of DIC.

4. Brief Description of the Drawings:

Fig. 1 is a diagram illustrating the results of Experimental Example 1 and relating the APC-PCI Complex (CIC) measured by the method of the present invention with the absorbance at the wavelength of 490 nm.

Fig. 2 is a diagram illustrating the results of Experimental Example 2, with the measured values of the APC-PCI Complex (CIC) in the healthy adult subjects and the patients with various disorders.

Fig. 3 is a diagram illustrating the results of Experimental Example 3 and following up the measured values of FDP, PCI, PC and APC-PCI Complex (CIC) with the elapse of time in an acute anterior myeloic leukemia patient.

Fig. 1

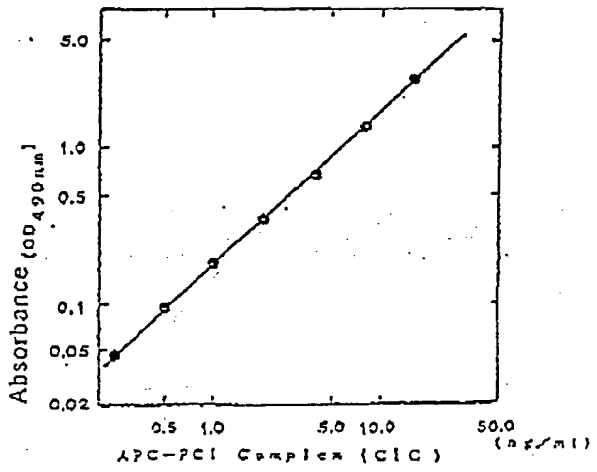

Fig. 2

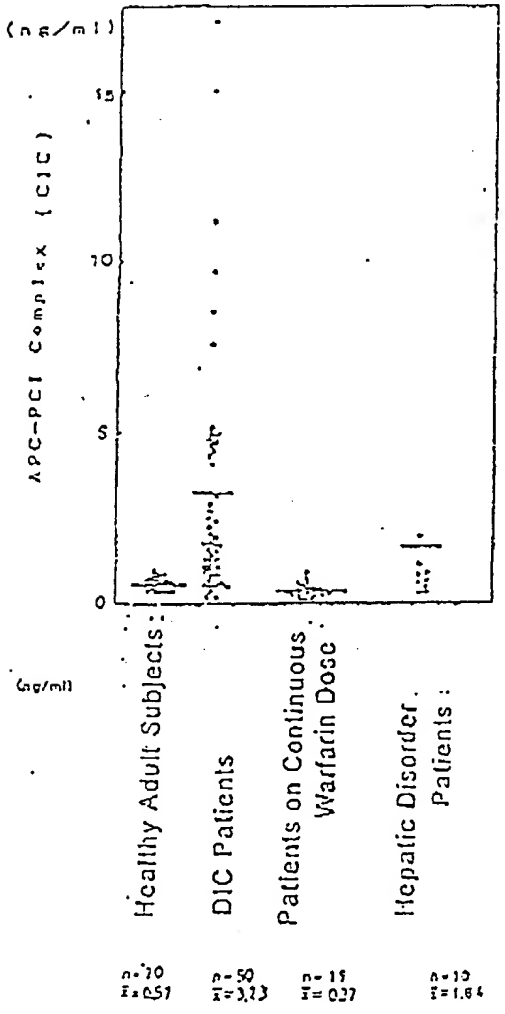

Fig. 3